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Testing Protocol

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Supplemental Assay Method for the Titration of Canine  
Parvovirus in Cell Culture

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Supplemental Assay Method for the Titration of Canine Parvovirus in Cell Culture

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## 1. Introduction

This Supplemental Assay Method (SAM) describes an *in vitro* test method for assaying modified-live canine parvovirus (CPV) vaccines for viral content. The method uses the Crandall feline kidney (CRFK) cell line as the test system. CPV endpoint is determined by staining inoculated cell cultures by an indirect fluorescent antibody (IFA) staining method.

## 2. Materials

### 2.1 Equipment/instrumentation

Equivalent equipment or instrumentation may be substituted for any brand name listed below.

2.1.1 Incubator,  $36^{\circ} \pm 2^{\circ}\text{C}$ , high humidity,  $5\% \pm 1\% \text{CO}_2$  (Model 3336, Forma Scientific Inc.)

2.1.2 Incubator aerobic (Model 2, Precision Scientific)

2.1.3 Water bath

2.1.4 Microscope, ultraviolet (UV) light (Model BH2, Olympus America)

2.1.5 Vortex mixer (Vortex-2 Genie, Model G-560, Scientific Industries)

2.1.6 Micropipettor, tips, and/or motorized microliter pipette or equivalent and tips

2.1.7 Microscope slide glass staining dish with rack (glass staining dish)

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## 2.2 Reagents/supplies

Equivalent reagents or supplies may be substituted for any brand name listed below. All reagents and supplies must be sterile.

**2.2.1** CPV Reference, KB5 strain [available from the Center for Veterinary Biologics (CVB)]

**2.2.2** CRFK cell culture, free of extraneous agents as tested by the Code of Federal Regulations, Title 9 (9 CFR)

**2.2.3** Feline panleukopenia virus antiserum (FPV Antiserum) or Canine Parvovirus antiserum (CPV Antiserum) [available from CVB]

**2.2.4** Minimum essential medium (MEM)

**2.2.4.1** 9.61 g MEM with Earles salts without bicarbonate

**2.2.4.2** 1.1 g sodium bicarbonate ( $\text{NaHCO}_3$ )

**2.2.4.3** Dissolve with 900 ml deionized water (DW).

**2.2.4.4** Add 5.0 g lactalbumin hydrolysate or edamine to 10 ml DW. Heat to  $60^\circ \pm 2^\circ\text{C}$  until dissolved. Add to **Section 2.2.4.3** with constant mixing.

**2.2.4.5** Q.S. to 1000 ml with DW; adjust pH to 6.8-6.9 with 2N hydrochloric acid (HCl).

**2.2.4.6** Sterilize through a 0.22- $\mu\text{m}$  filter.

**2.2.4.7** Aseptically add 50  $\mu\text{g/ml}$  gentamicin sulfate

**2.2.4.8** Store at  $2^\circ - 7^\circ\text{C}$ .

**2.2.5** Growth Medium

**2.2.5.1** 920 ml of MEM

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**2.2.5.2** Aseptically add:

1. 70 ml gamma-irradiated fetal bovine serum (FBS)
2. 10 ml L-glutamine (200 mM)

**2.2.6** Dulbecco's phosphate buffered saline (DPBS)

**2.2.6.1** 8.0 g sodium chloride (NaCl)

**2.2.6.2** 0.2 g potassium chloride (KCl)

**2.2.6.3** 0.2 g potassium phosphate, monobasic, anhydrous ( $\text{KH}_2\text{PO}_4$ )

**2.2.6.4** 0.1 g magnesium chloride, hexahydrate ( $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ )

**2.2.6.5** Dissolve reagents with 900 ml DW.

**2.2.6.6** Dissolve 1.03 g sodium phosphate, dibasic, anhydrous ( $\text{Na}_2\text{HPO}_4$ ) with 10 ml DW, heat to  $60^\circ \pm 2^\circ\text{C}$  until dissolved; add to **Section 2.2.6.5** with constant mixing.

**2.2.6.7** Dissolve 0.1 g calcium chloride, anhydrous ( $\text{CaCl}_2$ ) with 10 ml DW; add slowly to **Section 2.2.6.6** to avoid precipitation.

**2.2.6.8** Q.S. to 1000 ml with DW, adjust pH to 7.0-7.3 with 2N HCl.

**2.2.6.9** Sterilize through a 0.22- $\mu\text{m}$  filter.

**2.2.7** Cell culture glass slides, 8-well (Lab-Tek® Slides)

**2.2.8** Polystyrene tubes, 12 x 75-mm

**2.2.9** Appropriate anti-species IgG (H&L) fluorescein isothiocyanate labeled conjugate (Anti-species Conjugate)

**2.2.10** 100% acetone

**2.2.11** Syringe, 1-ml and needle, 20-gauge x 1 1/2-inch

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2.2.12 Pipette-Aid

2.2.13 Disposal transfer pipette, 4.6-ml

3. Preparation for the test

3.1 Personnel qualifications/training

Personnel shall have experience in the preparation and maintenance of cell culture as well as in the propagation of animal viruses and the quantitation of virus infectivity by the IFA staining method.

3.2 Preparation of equipment/instrumentation

3.2.1 On the day of inoculation, set a water bath at  $36^{\circ} \pm 2^{\circ}\text{C}$ .

3.2.2 On the day of the IFA test, prepare a humidity chamber in the aerobic incubator by filling a pan in the bottom with DW.

3.3 Preparation of reagents/control procedures

3.3.1 Preparation of CRFK Slides

3.3.1.1 Cells are prepared from healthy, confluent CRFK cells, that are maintained by passing every 3 to 4 days. On the day of test initiation, add 0.4 ml/well of  $10^{5.2}$  to  $10^{5.4}$  cells/ml diluted in Growth Media into all wells of a Lab-Tek® slide. Prepare sufficient Lab-Tek® Slides to allow 25 wells for controls and 20 wells for each Test Vaccine. Incubate at  $36^{\circ} \pm 2^{\circ}\text{C}$  in a  $\text{CO}_2$  incubator. These become the CRFK Slides.

3.3.1.2 Use seeded CRFK Slides within 4 hours.

3.3.2 Preparation of the CPV Working Reference

3.3.2.1 On the day of inoculation, rapidly thaw a vial of CPV Reference in a  $36^{\circ} \pm 2^{\circ}\text{C}$  water bath.

3.3.2.2 Dispense 1.8 ml of MEM into each of 7, 12 x 75-mm polystyrene tubes labeled  $10^{-1}$  through  $10^{-7}$ .

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**3.3.2.3** Transfer 200  $\mu$ l of the CPV Reference to the tube labeled  $10^{-1}$ ; discard pipette tip. Mix by vortexing.

**3.3.2.4** Using a new pipette tip, transfer 200  $\mu$ l from the  $10^{-1}$  labeled tube (**Section 3.3.2.3**) to the  $10^{-2}$  tube; discard pipette tip. Mix by vortexing.

**3.3.2.5** Repeat **Section 3.3.2.4** for each subsequent dilution, transferring 200  $\mu$ l from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

**3.3.3 Preparation of Working FPV or CPV Antiserum**

On the day of the IFA test, dilute FPV or CPV Antiserum in DPBS to the IFA working dilution on the Center for Veterinary Biologics (CVB) Reagent Data Sheet or as determined for that specific antiserum.

**3.3.4 Preparation of Working Anti-species Conjugate**

On the day of the IFA test, dilute Anti-species Conjugate in DPBS to the working dilution according to the manufacturer's recommendations.

**3.4 Preparation of the sample**

**3.4.1** The initial test of a Test Vaccine will be with a single vial (a single sample from 1 vial). On the day of inoculation, rehydrate a vial of the Test Vaccine by transferring 1.0 ml for a 1-ml-dose vaccine, 0.5 ml for 1/2-ml-dose vaccines, etc., of the provided diluent into the vial containing the lyophilized Test Vaccine. Use a sterile 1.0-ml syringe and an 18-gauge x 1 1/2-inch needle; mix by vortexing. Incubate for  $15 \pm 5$  minutes at room temperature.

**3.4.2** Dispense 1.8 ml MEM into each of 6, 12 x 75-mm polystyrene tubes labeled  $10^{-1}$  through  $10^{-6}$ .

**3.4.3** With a micropipettor, transfer 200  $\mu$ l from the rehydrated Test Vaccine vial to the tube labeled  $10^{-1}$ ; discard the pipette tip. Mix by vortexing.

**3.4.4** Transfer 200  $\mu$ l from the  $10^{-1}$  labeled tube (**Section 3.4.3**) to the  $10^{-2}$  tube; discard the pipette tip. Mix by vortexing.

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**3.4.5** Repeat **Section 3.4.4** for each subsequent dilution, transferring 200  $\mu$ l from the previous dilution to the next dilution tube until the tenfold dilution series is completed.

**4. Performance of the test**

**4.1** Inoculate 5 wells/dilution of a CRFK Slide with 100  $\mu$ l/well from dilutions  $10^{-6}$  through  $10^{-3}$  of the Test Vaccine. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g.,  $10^{-6}$  through  $10^{-3}$ ).

**4.2** Inoculate 5 wells/dilution of a CRFK Slide with 100  $\mu$ l/well, from dilutions  $10^{-6}$  through  $10^{-3}$  of the CPV Working Reference. Tip changes are not necessary between each dilution in a series if pipetting from the most dilute to the most concentrated within that series (e.g.,  $10^{-6}$  through  $10^{-3}$ ).

**4.3** Five uninoculated wells serve as a Negative Cell Control.

**4.4** Incubate CRFK Slides in a  $36^{\circ} \pm 2^{\circ}\text{C}$   $\text{CO}_2$  incubator for 5 days  $\pm$  1 day.

**4.5** Following incubation, decant the media from the CRFK Slide and remove the plastic walls by twisting them away from the CRFK Slide, leaving the gasket attached to the slide.

**4.6** Place the CRFK Slides in a slide rack; place the rack in a glass staining dish filled with DPBS. Let stand for  $15 \pm 5$  minutes at room temperature.

**4.7** Discard the DPBS; fix the CRFK Slides in 100% acetone for  $15 \pm 5$  minutes at room temperature. Remove and allow to air dry.

**4.8** Pipette  $75 \pm 25$   $\mu$ l of the Working FPV Antiserum into each well of the CRFK Slides. Incubate for  $30 \pm 5$  minutes in the aerobic incubator at  $36^{\circ} \pm 2^{\circ}\text{C}$ .

**4.9** Wash per **Section 4.6**.

**4.10** Pipette  $75 \pm 25$   $\mu$ l of the Working Anti-species Conjugate into each well of the CRFK Slides. Incubate for  $30 \pm 5$  minutes in the aerobic incubator at  $36^{\circ} \pm 2^{\circ}\text{C}$ .

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**4.11** Wash per **Section 4.6.** Discard the DPBS.

**4.12** Rinse the CRFK Slides with DW; allow to air dry.

**4.13** Read at 100-200X magnification with a UV-light microscope; examine the cell monolayer for typical CPV apple-green nuclear fluorescence.

**4.13.1** Wells containing 1 or more cells with specific CPV fluorescence are considered to be positive.

**4.13.2** Results are recorded as number of IFA positive wells versus total number of wells examined for each dilution of a Test Vaccine and the CPV Working Reference.

**4.14** Calculate the 50% fluorescent antibody infective dose (FAID<sub>50</sub>) of the Test Vaccine and the CPV Working Reference using the method of Spearman-Kärber as modified by Finney.

Example:

10<sup>-3</sup> dilution of Test Vaccine = 5/5 wells IFA positive  
10<sup>-4</sup> dilution of Test Vaccine = 4/5 wells IFA positive  
10<sup>-5</sup> dilution of Test Vaccine = 2/5 wells IFA positive  
10<sup>-6</sup> dilution of Test Vaccine = 0/5 wells IFA positive

Spearman-Kärber formula:

**Test Vaccine Titer = (X - d/2 + [d • S]) where:**

**X** = log<sub>10</sub> of dilution with all wells IFA positive (3)

**d** = log<sub>10</sub> of dilution factor (1)

**S** = sum of proportions of wells IFA positive for all dilutions tested:

$$\frac{5}{5} + \frac{4}{5} + \frac{2}{5} + \frac{0}{5} = \frac{11}{5} = 2.2$$

**Test Vaccine titer = (3 - 1/2) + (1 • 2.2) = 4.7**

Adjust the titer to the Test Vaccine dose as follows:

**A.** divide the **Test Vaccine Dose** by the **Inoculation Dose**

**Test Vaccine Dose** = manufacturer's recommended vaccination dose (for this CPV test vaccine, the recommended dose is 1 ml)

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**Inoculation Dose** = amount of diluted Test Vaccine added to each well of the Test Slide (for this CPV test vaccine, the inoculation dose is 0.1 ml)

$$\frac{1 \text{ ml dose}}{0.1 \text{ ml inoculum}} = 10$$

**B.** calculate  $\log_{10}$  of value in **A** and add it to the **Test Vaccine titer** as illustrated below:

$$\text{Log of } 10 = 1.0$$

$$\text{Test Vaccine titer} = 4.7 + 1.0 = 5.7$$

Therefore the titer of the **CPV Test Vaccine** is  $10^{5.7}$  FAID<sub>50</sub>/ml.

**5. Interpretation of the test results**

**5.1 Validity requirements:**

**5.1.1** The calculated FAID<sub>50</sub> titer of the CPV Working Reference must fall within plus or minus 2 standard deviations ( $\pm 2$  SD) of its mean titer, as established from a minimum of 10 previously determined titers.

**5.1.2** The lowest inoculated dilution of the CPV Working Reference must exhibit a 100% positive IFA reaction (5/5). If an endpoint is not reached (1 or more wells are IFA positive at the highest dilution), the titer is expressed as "greater than or equal to" the calculated titer. If an endpoint is critical to testing, the highest (most dilute) must exhibit no positive IFA reaction (0/5).

**5.1.3** The Uninoculated Cell Control must not exhibit any cytopathic effect, specific CPV fluorescence, or cloudy media that would indicate contamination.

**5.2** If the validity requirements are not met, then the assay is considered a **NO TEST** and may be retested without prejudice.

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**5.3** If the validity requirements are met and the titer of the Test Vaccine is greater than or equal to the titer contained in the Animal and Plant Health Inspection Service (APHIS) filed Outline of Production for the product under test, the Test Vaccine is considered **SATISFACTORY**.

**5.4** If the validity requirements are met, but the titer of the Test Vaccine is less than the required minimum titer contained in the APHIS filed Outline of Production for the product under test, the Test Vaccine may be retested in accordance with the 9 CFR, Part 113.8.

## **6. Report of test results**

Report results as the FAID<sub>50</sub> per dose of the Test Vaccine.

## **7. References**

**7.1** Code of Federal Regulations, Title 9, Part 113.317, U.S. Government Printing Office, 2004.

**7.2** Cottral, GE, (Ed.), 1978. *Manual of standardized methods for veterinary microbiology*. Comstock Publishing Associates, Ithaca, NY. pg. 731.

**7.3** Finney, DJ, 1978. *Statistical methods in biological assay*. Griffin, London. 3rd edition, pp. 394-401.

## **8. Summary of revisions**

This document was revised to clarify the practices currently in use in the Center for Veterinary Biologics and to provide additional detail. While no significant changes impacting the test were made from the previous protocol, the following changes have been made:

- **2.1.2, 2.1.3** The temperature setting has been removed.
- **2.1.6, 2.2.12, 2.2.13** Equipment has been added.
- **2.2.3, 3.3.3** The reagent name has been corrected to "or CPV antiserum".
- **2.2.4.2** The amount of sodium bicarbonate (NaHCO<sub>3</sub>) has been changed from 2.2 g to 1.1 g.

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- 2.2.4.7 Penicillin and streptomycin have been deleted.
- 2.2.9, 3.3.4, 4.10 The conjugate name has been corrected to Anti-Species IgG.
- 3.1 Personnel experience has been clarified.
- 3.3.1 The cell count has been clarified.
- 4.2 Inoculation of the Reference has been clarified.
- 4.4 The incubation has been changed from hours to days.
- 4.9, 4.11 The wash step has been corrected.
- 4.13.2 The recording step has been corrected.
- 4.14 Additional steps have been added to clarify the titer calculations by the Spearman-Kärber formula.
- 5.1.2 Recording the rate of positive reaction for validity requirements.
- "Reference and Reagent Sheet" has been changed to "Reagent Data Sheet" throughout the document.
- The refrigeration temperatures have been changed from  $4^{\circ}\pm 2^{\circ}\text{C}$  to  $2^{\circ}\text{--}7^{\circ}\text{C}$ . This reflects the parameters established and monitored by the Rees system.
- The word "chamber" has been changed to "well" throughout the document.
- The footnotes have been deleted with any pertinent references now noted next to the individual items.
- "Test Serial" has been changed to "Test Vaccine" throughout the document.